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Genomic Sequence and Phylogenetic Analysis of *Culex* Flavivirus, an Insect-Specific Flavivirus, Isolated From *Culex pipiens* (Diptera: Culicidae) in Iowa

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Abstract

Adult mosquitoes (Diptera: Culicidae) were collected in 2007 and tested for specific viruses, including West Nile virus, as part of the ongoing arbovirus surveillance efforts in the state of Iowa. A subset of these mosquitoes (6,061 individuals in 340 pools) was further tested by reverse transcription-polymerase chain reaction (RT-PCR) using flavivirus universal primers. Of the 211 pools of *Culex pipiens* (L.) tested, 50 were positive. One of 51 pools of *Culex tarsalis* Coquillett was also positive. The flavivirus minimum infection rates (expressed as the number of positive mosquito pools per 1,000 mosquitoes tested) for *Cx. pipiens* and *Cx. tarsalis* were 10.3 and 1.2, respectively. Flavivirus RNA was not detected in *Aedes triseriatus* (Say) (52 pools), *Culex erraticus* (Dyar & Knab) (25 pools), or *Culex territans* Walker (one pool). Sequence analysis of all RT-PCR products revealed that the mosquitoes had been infected with *Culex* flavivirus (CxFV), an insect-specific virus previously isolated in Japan, Indonesia, Texas, Mexico, Guatemala and Trinidad. The complete genome of one isolate was sequenced, as were the envelope protein genes of eight other isolates. Phylogenetic analysis revealed that CxFV isolates from the United States (Iowa and Texas) are more closely related to CxFV isolates from Asia than those from Mexico, Guatemala, and Trinidad.

Keywords

Flavivirus; *Culex* flavivirus; mosquito; insect; surveillance

The majority of viruses in the genus *Flavivirus* are arthropod-borne viruses that are transmitted between vertebrate hosts by mosquitoes or ticks (ICTV 2005). Other viruses in this genus have a vertebrate host, but no known arthropod vector. Viruses in the final group replicate in mosquitoes but have no apparent vertebrate host and are therefore assumed to be insect-specific. Three insect-specific flaviviruses have been identified, and these are *Culex* flavivirus (CxFV), cell fusing agent virus (CFAV) and Kamiti River virus (KRV) (Stollar and Thomas 1975, Crabtree et al. 2003, Sang et al. 2003, Hoshino et al. 2007). In addition, flavivirus-related DNA known as cell silent agent is integrated into the genomes of some *Aedes* spp. mosquitoes

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(Crochu et al. 2004). Phylogenetically, insect-specific flaviviruses occupy the most divergent lineage, suggesting that they are primordial flaviviruses that emerged before the other members of this genus (Cook and Holmes 2006, Hoshino et al. 2007).

The first insect-specific flavivirus to be discovered was CFAV, originally isolated from *Aedes aegypti* (L.) cell cultures in 1975 (Stollar and Thomas 1975, Cammisa-Parks et al. 1992), and later discovered in natural populations of *Ae. aegypti*, *Aedes albopictus* (Skuse), and *Culex* spp. mosquitoes in Puerto Rico, and *Ae. aegypti* in Thailand (Cook et al. 2006, Kihara et al. 2007). In Puerto Rico, CFAV was isolated from both female and male mosquitoes suggesting that the virus can be *transovarially* transmitted in nature (Cook et al. 2006). CFAV causes cytopathic effect (CPE), including massive syncytium formation, in *Ae. albopictus* (C6/36) cells, but it does not replicate in mice or in any of the vertebrate cell lines that have been tested (Stollar and Thomas 1975, Crabtree et al. 2003). KRV, the second insect-specific flavivirus to be discovered, was isolated from immature *Aedes macintoshi* Marks collected in Kenya in 1999 (Crabtree et al. 2003, Sang et al. 2003). KRV replicates in *Ae. albopictus*, *Ae. aegypti* and *Aedes pseudoscutellaris* (Theobald) cell cultures, but not in mice or vertebrate cell cultures. KRV causes CPE in C6/36 cells but not *Ae. aegypti* and *Ae. pseudoscutellaris* cells. The 3' untranslated region (UTR) of KRV is unusual; it is nearly twice as long as the 3'UTRs of all other flaviviruses and seems to have formed as the result of an almost complete duplication of a primordial KRV 3'UTR (Gritsun and Gould 2006).

CxFV was first isolated from *Culex* spp. mosquitoes collected in Japan and Indonesia in 2003 and 2004 (Hoshino et al. 2007). Nine isolations were made from *Cx. pipiens* ($n = 6$), *Culex quinquefasciatus* Say ($n = 2$), and *Culex tritaeniorhynchus* Giles ($n = 1$). Isolations were made from both female and male mosquitoes. The authors observed that the isolates could replicate in C6/36 cells, but not in African green monkey kidney (Vero) or baby hamster kidney cells. Moderate CPE was periodically observed in C6/36 cells inoculated with CxFV that had been passed at least twice in cell culture. However, CPE was usually absent in cells infected with the original inoculum or virus passed once.

More recently, CxFV has been identified in the Western Hemisphere, with isolates obtained from Guatemala (Morales-Betoulle et al. 2008), the Yucatan Peninsula of Mexico (Farfan-Ale et al. 2009), Trinidad, Texas (Kim et al. 2009), and Colorado (B. G. Bolling, personal communication). In Guatemala, CxFV was detected by reverse transcription-polymerase chain reaction (RT-PCR) in eight of 210 pools of female *Cx. quinquefasciatus* collected in 2006 (Morales-Betoulle et al. 2008). The CxFV minimum infection rate (MIR), expressed as the number of positive mosquito pools per 1,000 mosquitoes tested, was 4.7. This strain of CxFV (designated Izabal) did not cause CPE in C6/36 cells. In Mexico, CxFV was detected by RT-PCR in 145 of 210 pools (MIR 20.8) of *Cx. quinquefasciatus* collected in 2007 (Farfan-Ale et al. 2009). Like the Izabal strain, CxFV strains from Mexico do not induce CPE in C6/36 cells. The virus was detected in both female and male mosquitoes. In Texas, CxFV was detected in 37 of 388 pools of female *Cx. quinquefasciatus* or *Culex restuans* Theobald collected in 2008 (Kim et al. 2009). Some CxFV strains from Texas produced marked CPE and syncytia formation in C6/36 cells, whereas others did not. Phylogenetic analyses of the envelope protein (E) genes have shown that CxFV can be separated into two distinct clades. One clade contains CxFV isolates from Texas and Asia, and the second clade contains CxFV isolates from Latin America and the Caribbean (Kim et al. 2009).

In the current study, mosquitoes collected in Iowa in 2007 were examined by RT-PCR using flavivirus-specific primers. Of the 340 pools tested, 51 were positive for CxFV RNA. A subset of mosquito homogenates was processed by virus isolation in C6/36 cells, and 12 isolates were obtained. The genomic RNA of one isolate and the E genes of another eight isolates were sequenced. This report presents the sequence and phylogenetic data.

Materials and Methods

Study Sites and Collection Methods

Collections were made in multiple sites in 13 counties in the state of Iowa from May through October 2007 (Fig. 1). Mosquitoes were sampled using CDC light, gravid, and mosquito magnet traps set from dusk until the next morning. Mosquitoes were transported on ice to the Medical Entomology Laboratory at Iowa State University (ISU), where they were sorted on chill tables into groups of up to 50 according to species, sex, date, and study site, and then stored at -80°C . Due to the difficulty in morphologically distinguishing adult *Cx. pipiens* and *Cx. restuans* individuals, these mosquitoes were grouped and are referred to as the *Cx. pipiens* group as described previously (DeGroot et al. 2007, Sucaet et al. 2008).

Virus Identification by RT-PCR Sequencing

Mosquitoes were homogenized in 1.8 ml of diluent (minimum essential media containing 10% fetal bovine serum, L-glutamine, penicillin, streptomycin, and Amphotericin B) as described previously (Farfan-Ale et al. 2009). Total RNA was extracted from mosquito homogenates using the QIAamp viral RNA extraction kit (QIAGEN, Valencia, CA), and analyzed by RT-PCR using the flavivirus-specific primers: FU2: 5'-GCT GAT GAC ACC GCC GGC TGG GAC AC-3' and cFD3: 5'-AGC ATG TCT TCC GTG GTC ATC CA-3', which target a 845-nt region of the NS5 gene (Kuno et al. 1998). Complementary DNAs were generated using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA), and PCRs were performed using *Taq* polymerase (Invitrogen) as described previously (Farfan-Ale et al. 2009). An aliquot of each PCR product was examined by electrophoresis, visualized with ethidium bromide, and extracted using the PureLink gel extraction kit (Invitrogen). Purified DNAs were sequenced using a 3730x1 DNA sequencer (Applied Biosystems, Foster City, CA) at ISU.

Virus Isolation in C6/36 Cells

An aliquot (100 μl) of selected mosquito homogenates was added to 2 ml of Liebovitz's L15 medium (Invitrogen) supplemented with 2% fetal bovine serum, L-glutamine, penicillin, streptomycin, and Amphotericin B. Samples were filtered and inoculated onto subconfluent monolayers of *Ae. albopictus* C6/36 cells in 75-cm² flasks. Cells were incubated for at least 1 h at room temperature on an orbital shaker to allow attachment of the virus. Another 12 ml of L15 maintenance medium was added to each flask, and cells were incubated at 28°C for 7 d. After two additional blind passages, supernatants were collected and tested for the presence of CxFV.

CxFV Genomic Sequencing

Primers for the RT-PCR amplification and sequencing of CxFV were designed using the genomic sequences of a Japanese CxFV isolate (strain NIID-21) and a Mexican CxFV isolate (strain CxFV-Mex07) (Hoshino et al. 2007, Farfan-Ale et al. 2009). The resulting PCR products were sequenced and used to design additional primers. In total, 24 pairs of overlapping primers were used, and primer sequences are available upon request. The extreme 5' and 3' ends of the CxFV genome were determined by 5' rapid amplification of cDNA ends (RACE) and 3' RACE, respectively, as described previously (Farfan-Ale et al. 2009).

Results

As part of the ongoing arbovirus surveillance efforts in Iowa, mosquitoes are collected throughout the state each year and select species are assayed by RT-PCR using primers specific for West Nile, St. Louis encephalitis, La Crosse and/or Western equine encephalitis viruses. A subset of mosquitoes that had tested negative for these viruses was randomly selected for

the current study. Overall, 6,061 mosquitoes (340 pools) collected in 13 Iowa counties from May through October 2007 were chosen. There were 4,847 *Cx. pipiens* group (211 pools), 859 *Cx. tarsalis* Coquillett (51 pools), 239 *Ae. triseriatus* (Say) (52 pools), 115 *Culex erraticus* (Dyar & Knab) (25 pools) and one *Cx. territans* Walker (one pool) (Table 1). All pools were comprised of female mosquitoes.

Mosquito pools were screened by RT-PCR using flavivirus-specific primers, and 51 pools tested positive (Table 1). Of these, 50 pools consisted of *Cx. pipiens* group and one pool consisted of *Cx. tarsalis*. The flavivirus MIRs for *Cx. pipiens* group and *Cx. tarsalis* were 10.3 and 1.2, respectively. Because of the considerable variation in pool sizes, bias corrected maximum likelihood estimation (MLE) values also were calculated using the PooledInfRate statistical software package (Biggerstaff 2006). The resulting MIR and MLE values were consistently very similar; therefore, MLE data are not shown. The flavivirus MIR for *Cx. pipiens* group showed considerable geographic variation (Table 2). For the five counties from which >200 *Cx. pipiens* group females were collected, the flavivirus MIR was 3.4–28.3. Flavivirus RNA was not detected in any *Ae. triseriatus*, *Cx. erraticus*, or *Cx. territans*. All PCR products were partially sequenced and shown to correspond to CxFV. Pairwise alignments of the resulting sequences revealed that they share >98% identity. The mosquitoes positive for CxFV RNA were collected from July through October (Table 3). The monthly MIRs for CxFV in *Cx. pipiens* group collected in this time period ranged from 7.2 to 61.0. CxFV was not detected in any mosquitoes collected in May or June.

Homogenates from 12 pools containing CxFV RNA were filtered and an aliquot of each was inoculated on C6/36 cells. One week later, an aliquot of each supernatant was inoculated onto fresh monolayers of C6/36 cells. In total, three blind passages were performed. Mild CPE and/or clumping of cells were observed in all cell cultures with the exception of mock-infected control cultures. CxFV RNA was detected by RT-PCR in all supernatants collected after the third blind passage (except for the control cultures). Taken together, these data indicate the CxFV had been isolated from all 12 homogenates tested.

The complete genomic sequence of one isolate (designated CxFV-Iowa07) was determined using a combination of RT-PCR, 5' and 3' RACE (GenBank accession FJ663034). The genomic RNA contains a single 10,089-nt open reading frame that is flanked by 5' and 3'UTRs of 91 and 654 nt, respectively. In the 5' RACE experiments, 12 cDNA clones were analyzed, and the length and sequence of all clones were identical. Genomic sequence data are available for four other CxFV isolates; two isolates are from Japan (strains Tokyo and NIID-21), one isolate is from Mexico (strain CxFV-Mex07), and one isolate is from Texas (strain TX 24518) (Hoshino et al. 2007, Farfan-Ale et al. 2009, Kim et al. 2009). Pairwise alignment of the CxFV-Iowa07 genomic sequence to the other CxFV genomic sequences revealed that it is most closely related to isolates from Japan and Texas (97.6–98.9% identical). There is 90.9% identity between the genomes of CxFV-Iowa07 and CxFV-Mex07. The predicted amino acid sequence of the CxFV-Iowa07 polyprotein precursor also is more closely related to the polyprotein precursors of CxFV isolates from Japan and Texas (≥99.0% identical, ≥99.4% similar) than to the isolate from Mexico (97.0% identical, 98.4% similar).

A phylogenetic tree was constructed with Bayesian methods using the complete genomic sequence of CxFV-Iowa07, and the genomic sequences of 24 other flavivirus isolates (Fig. 2). Phylogenetic trees also were generated using neighbor-joining (NJ), maximum parsimony (MP), and maximum likelihood (ML) methods. In the Bayesian tree, CxFV-Iowa07 shares a close phylogenetic relationship with CxFV isolates from Japan, Texas, and Mexico, consistent with the identity/similarity estimates. These viruses, together with the other insect-specific flaviviruses (CFAV and KRV) comprise a distinct clade (denoted as I). The posterior support for this topological arrangement is 100%. The other viruses separate into two additional clades.

Clade II contains tick/vertebrate viruses and clade III contains mosquito/vertebrate viruses. Viruses with no known vector are present in both clades II and III. This topological arrangement is consistent to that observed in several other flavivirus phylogenetic studies (Cook and Holmes 2006). The ML and NJ trees were identical to the Bayesian tree, but the MP tree placed Modoc virus between the insect-specific flaviviruses and the other flaviviruses (data not shown).

After the completion of the CxFV-Iowa07 genomic sequencing experiments, the E genes of eight other CxFV isolates from Iowa were also sequenced (GenBank accessions FJ663026–FJ663033). This region comprises 1,281 nucleotides and corresponds to nucleotides 938–2,218 of the genomic RNA of the CxFV-Iowa07 strain. Pairwise alignments of these sequences revealed that they share 97.7 to 99.9% identity. The E gene sequences of 21 other isolates are also available in the GenBank database (Hoshino et al. 2007, Morales-Betoulle et al. 2008, Farfan-Ale et al. 2009, Kim et al. 2009). These isolates are from Japan ($n = 9$), Indonesia ($n = 1$), Texas ($n = 7$), Mexico ($n = 1$), Guatemala ($n = 1$), and Trinidad ($n = 2$). The nucleotide sequence of the E gene of CxFV-Iowa07 was aligned to the homologous regions of a representative isolate from each of these geographic locations. As observed with the genomic sequence alignments, this analysis demonstrated that CxFV-Iowa07 is most closely related to CxFV isolates from Japan, Indonesia, and Texas ($\geq 97.7\%$ identity) (Table 4). In contrast, the E gene of CxFV-Iowa07 shares no more than 90.1% identity to the homologous regions of CxFV isolates from Mexico, Guatemala, and Trinidad. Similarly, the deduced amino acid sequence of the E protein of CxFV-Iowa07 shares greater identity and similarity to the homologous regions of CxFV isolates from Japan, Indonesia, and Texas than to those from Mexico, Guatemala, and Trinidad.

A phylogenetic tree was constructed with Bayesian methods by using the E gene sequences of 30 CxFV isolates, including nine isolates from Iowa (Fig. 3). Phylogenetic trees also were generated using NJ, MP, and ML methods. In the Bayesian tree, the CxFV isolates formed two distinct clades (denoted as clade 1 and 2). Clade 1 is composed of CxFV isolates from Mexico, Guatemala, and Trinidad (denoted as the Latin American/Caribbean clade). The posterior support for this clade is 0.87. Clade 2 is composed of CxFV isolates from Iowa, Texas, Japan, and Indonesia (denoted as the U.S./Asian clade). The posterior support for this clade is 0.65. Clade 2 is further separated into three nested clades (denoted as 2a to 2c). Eight CxFV isolates from Iowa grouped in clade 2c, along with all the CxFV isolates from Texas. One CxFV isolate from Iowa (CxFV-Iowa07) clustered in 2b, along with several isolates from Japan. Clade 2a was composed of CxFV isolates from Japan and Indonesia. Whereas these three subclades have reasonably high posterior support (0.98, 0.94, and 0.77, respectively), their relationship is less certain. The ML tree showed the same topological features as the Bayesian tree. In the MP tree, clades 2a and 2b are nearest neighbors, rather than 2b and 2c. The Iowa/Texas sequences do not form a monophyletic clade in the NJ tree but instead branch off tightly just ancestral to the 2a/2b cluster (data not shown).

The placement of CxFV-Iowa07 in the phylogenetic tree constructed using E gene sequences is interesting. The isolate clusters in clade 2b; all other isolates from the United States belong to clade 2c. Nucleotide sequence alignments revealed that CxFV-Iowa07 and all other members of clade 2b share 19 mutations that are not found in any isolates in clade 2c. These mutations are located in the following nucleotide positions in the CxFV-Iowa genome: 1,108, 1,114, 1,276, 1,297, 1,318, 1,438, 1,534, 1,615, 1,645, 1,692, 1,786, 1,825, 1,840, 2,050, 2,065, 2,096, 2,143, 2,146 and 2,185. Ten of these mutations are also present in all isolates in clade 2a. All isolates in the Latin American/Caribbean clade share 88 mutations that are not found in any isolates in the United States/ Asian clade (data not shown).

Discussion

We report the isolation and sequence determination of CxFV isolates from *Culex* spp. mosquitoes collected in Iowa. CxFV also was recently isolated from mosquitoes collected in Japan, Indonesia, Guatemala, Mexico, Trinidad, and Texas (Hoshino et al. 2007, Morales-Betoulle et al. 2008, Farfan-Ale et al. 2009, Kim et al. 2009). In addition, CFAV was recently discovered in natural mosquito populations in Puerto Rico and Thailand (Cook et al. 2006, Kihara et al. 2007), and KRV was isolated from mosquitoes collected in Africa (Crabtree et al. 2003, Sang et al. 2003). Together, these data indicate that insect-specific viruses are common and widely distributed in nature. One explanation as to why CxFV was not identified until recently is because it causes minimal (if any) CPE in mosquito cells and does not replicate in mammalian cells. As a result, the virus could easily remain undetected in arbovirus surveillance studies in which mosquitoes are screened for viruses by suckling mouse brain inoculation or by virus isolation in Vero or C6/36 cells.

The phylogenetic analyses demonstrate that CxFV isolates from Iowa are most closely related to CxFV isolates from Asia and Texas. These findings are consistent with the E gene sequence alignments which reveal that CxFV isolates from Iowa share $\geq 97.7\%$ identity to isolates from Asia and Texas, but no more than 90.1% identity to those from Guatemala, Mexico, and Trinidad. It is surprising that CxFV isolates from Iowa have a closer genetic and phylogenetic relationship to isolates from the Eastern Hemisphere than to isolates from most of the Western Hemisphere locations mentioned above. One explanation is that CxFV has spread “horizontally” across the globe, possibly due to the displacement of mosquitoes by human activity (i.e., air and sea transportation) (Lounibos 2002). Despite these genetic differences, these isolates still represent a single viral species. According to Kuno and colleagues, a viral species is defined as a group of viruses with $>84\%$ nucleotide sequence identity among them (Kuno et al. 1998).

CxFV RNA was detected in mosquitoes collected from July through October but was not detected in any mosquitoes collected in May or June (Table 3). The absence of CxFV RNA in the May and June collections could reflect the lower numbers of *Culex* spp. mosquitoes collected in these months. Only 8.3% of the total number of *Cx. pipiens* group was collected in May and June. These findings also could reflect the lower numbers of *Culex* spp. mosquitoes collected at specific study sites during these months. For example, no *Cx. pipiens* were collected in Polk County in May. This county yielded the highest overall CxFV MIR when compared with all other counties from which >200 *Cx. pipiens* group females were collected. In Texas, CxFV was detected in mosquitoes from November to March but not April to August, even though mosquitoes were abundant at these times (Kim et al. 2009). Although this would seem to contradict the apparent transmission cycle of CxFV, these data indicate that CxFV infection could be seasonal in some areas.

Mosquitoes infected with one virus are often refractory or less susceptible to subsequent infection with closely related viruses due to the phenomenon of superinfection exclusion (Davey et al. 1979, Sundin and Beaty 1988). Thus, future research should be performed to determine whether mosquitoes infected with CxFV are resistant to subsequent infection with other flaviviruses. These experiments could lead to the development of novel strategies that block the transmission of medically important flaviviruses.

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Fig. 1.
Geographic locations of the Iowa counties used for mosquito collections, and the CxFV minimal infection rates for each county.

Fig. 2.

Phylogenetic analysis of the genomic sequence of a *Culex flavivirus* isolate from Iowa (CxFV-Iowa07). The displayed phylogeny was estimated by using the program MRBAYES, version 3.1 (Ronquist and Huelsenbeck 2003). The phylogenetic analysis is based on the complete genomic sequence of CxFV-Iowa07 and 24 other flavivirus isolates. An alignment without the Iowa and Texas CxFV sequences had been prepared by aligning the 5'UTR, polyprotein, and 3'UTR separately and then concatenating (Farfan-Ale et al. 2009). For the polyprotein, a protein alignment was back-translated to recover a nucleotide alignment. All alignments were done using the linsi utility of MAFFT (Minin et al. 2005), which was also used to profile align the CxFV isolates to the full genome alignment. The evolutionary model GTR+G+I was assumed

based on previous analysis (Farfan-Ale et al. 2009). Phyml was used for the ML tree (Guindon and Gascuel 2003), the Vanilla 1.2 frontend to PAL 1.2 (Drummond and Strimmer 2001) was used for the NJ tree (using the GTR+G model), and Phylip was used for the MP tree (Felsenstein 2004). An initial tree figure was obtained using midpoint rooting in NJplot (Perriere and Gouy 1996). Bootstrap support (out of 100) and posterior support for each branch are indicated. CxFV-Iowa07 is denoted in bold. GenBank accession numbers for sequences used in the phylogenetic analysis are as follows: Cell fusing agent virus (M91671), Culex flavivirus (strain CxFV-Iowa07 from Iowa; FJ663034), Culex flavivirus (strain Tokyo from Japan; AB262759), Culex flavivirus (strain CxFV-Mex07 from Mexico; EU879060), Culex flavivirus (strain TX 24518 from Texas; FJ502995), Dengue type 1 (U88536), Dengue type 2 (U87411), Dengue type 3 (AY099336), Dengue type 4 (AF326825), Entebbe bat (DQ837641), Japanese encephalitis (M18370), Kamiti River (AY149905), Langat (AF253419), Louping ill (Y07863), Modoc (AJ242984), Murray Valley encephalitis (AF161266), Omsk hemorrhagic fever (AY193805), Powassan (L06436), Sepik (DQ837642), St. Louis encephalitis (DQ525916), Tick-borne encephalitis (U27495), Usutu (AY453411), West Nile (DQ211652), Yellow fever (X03700), and Yokose (AB114858) viruses.

Fig. 3.

Phylogenetic analysis of the envelope protein genes of nine *Culex flavivirus* isolates from Iowa. The displayed phylogeny was estimated by using the program MRBAYES, version 3.1 (Ronquist and Huelsenbeck 2003). The phylogenetic analysis is based on the envelope protein genes of 31 flavivirus isolates (nine CxFV isolates from Iowa, 21 CxFV isolates from other geographical locations, and one isolate of Kamiti River virus). All nucleotide sequences were aligned using the MAFFT linsi utility. jModelTest supported the K80+G model under the BIC criterion and GTR+G using Akaike information criterion, but both models gave the same results for all methods, although the methods disagreed with each other (see text). ML, NJ, and MP trees were made as described in the Fig. 1 legend. The displayed tree is rooted by the Kamiti

River virus, which is not shown. The MrBayes consensus tree included several multifurcations. All other nodes, except two in the Texas cluster for lack of space, have bootstrap/posterior support indicated. CxFV isolates identified in this study are denoted in bold. GenBank accession numbers for sequences used in the phylogenetic analysis are as follows: CxFV-Iowa07 (FJ663034), IA 377 (FJ663031), IA 318 (FJ663030), IA 383 (FJ663028), IA 657 (FJ663033), IA 599 (FJ663032), IA 635 (FJ663029), IA 380 (FJ663027), IA 1064 (FJ663026), CxFV-Mex07 (EU879060), Hokkaido (AB262762), Izabal (EU805805), Mie (AB262767), Mie *pipiens* (AB262764), Morioka (AB262765), Narita (AB262760), NIID-21 (AB377213), Osaka (AB262763), Surabaya (AB262766), Tokyo (AB262759), Toyama (AB262761), Trinidad 11264 (FJ503002), Trinidad 11265 (FJ503003), TX-24284 (FJ502997), TX-24471 (FJ502998), TX-24516 (FJ502999), TX-24518 (FJ502995), TX-24519 (FJ502996), TX-24522 (FJ503000), TX-24559 (FJ503001), and Kamiti River virus (AY149905).

Table 1
Minimum infection rates for *Culex flavivirus* in mosquitoes from Iowa

Species	No. mosquitoes	No. pools	No. pools positive for CxFV RNA	CxFV MIR
<i>Cx. erraticus</i>	115	25	0	0
<i>Cx. tarsalis</i>	859	51	1	1.2
<i>Cx. territans</i>	1	1	0	0
<i>Cx. pipiens</i> group	4,847	211	50	10.3
<i>Ae. triseriatus</i>	239	52	0	0
Total	6,061	340	51	8.4

Table 2
Culex flavivirus minimum infection rates in *Cx. pipiens* group collected in different Iowa counties

County	No. <i>Cx. pipiens</i> group	No. <i>Cx. pipiens</i> group pools	No. pools positive for CxFV RNA	CxFV MIR
Allamakee	166	5	1	6.0
Black Hawk	8	8	0	0
Dubuque	142	10	3	21.1
Johnson	28	2	0	0
Kossuth	15	2	1	66.7
Linn	8	3	1	125.0
Lucas	1,182	39	5	4.2
Monona	51	7	1	19.6
Polk	495	42	14	28.3
Pottawattamie	160	4	0	0
Scott	1,182	28	4	3.4
Story	996	46	14	14.1
Woodbury	414	15	6	14.5
Total	4,847	211	50	10.3

Table 3
Seasonal prevalence of *Culex flavivirus* in *Cx. pipiens* group collected in Iowa

Mo	No. <i>Cx. pipiens</i> group	No. <i>Cx. pipiens</i> group pools	No. pools positive for CxFV RNA	CxFV MIR
May	34	4	0	0
June	368	21	0	0
July	946	48	17	18.0
Aug.	2,906	88	21	7.2
Sept.	511	38	7	13.7
Oct.	82	12	5	61.0
Total	4,847	211	50	10.3

Table 4
Nucleotide identity (top) and amino acid identity/similarity (bottom) between the envelope protein genes of representative *Culex* flavivirus isolates from different geographical locations

	Iowa	Guatemala	Indonesia	Japan	Mexico	Texas	Trinidad
Iowa	—	89.9	97.7	98.7	89.8	97.7	90.1
Guatemala	97.7/99.3	—	90.2	89.4	99.2	90.0	98.5
Indonesia	99.1/99.8	97.2/99.1	—	97.5	90.0	97.5	90.5
Japan	99.8/100.0	97.4/99.3	99.3/99.8	—	89.2	97.5	89.7
Mexico	97.7/99.3	100.0/100.0	97.2/99.1	97.4/99.4	—	89.8	98.5
Texas	99.3/99.8	97.4/99.1	99.3/99.5	99.5/99.8	97.4/99.1	—	90.0
Trinidad	97.7/99.1	99.5/99.8	97.2/98.8	97.4/99.1	99.5/99.8	97.4/98.8	—

Alignments were performed using the following CxFV isolates: CxFV-Iowa07 (Iowa), NIID-1 (Japan), Surabaya (Indonesia), TX 24518 (Texas), Trinidad 11264 (Trinidad), Izabal (Guatemala), and CxFV-Mex07 (Mexico).